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DESTRUCTION OF SPOILAGE AND PATHOGENIC BACTERIA BY HYDROSTATIC PRESSURE AND ELECTROPORATION IN COMBINATION WITH BIOPRESERVATIVES

PHASE II

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Escherichia coli O157:H7, *Salmonella typhimurium* ATCC 14028, *Listeria monocytogenes* Scott A, *Leuconostoc mesenteroides* Ly and *Lactobacillus sake* FM1 were evaluated for their sensitivity to either hydrostatic pressure (HP) or electroporation (EP) in combination with temperature, biopreservative and lysozyme. Both HP and EP caused viability loss and sublethal injury to bacterial cells. A combination of HP and mild temperature induced greater sublethal injury to cells. Sublethally-injured cells became sensitive to a biopreservative and lysozyme. By combining biopreservative and lysozyme, EP at 15 kV/cm for 30 μs caused viability loss of 3.0 logs in *Salmonella typhimurium* and 2.2 logs in *Escherichia coli*. The viability loss reached 4.3 logs for *Escherichia coli* and 10.9 logs for *Listeria monocytogenes* when cells were exposed to 30,000 lb/in^2 at 35°C in the presence of biopreservative and lysozyme.

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PREFACE

This study was conducted from October 1994 through September 1995 by Mssrs. Norasak Kalchayanand and Bibek Ray, University of Wyoming, under the supervision of Drs. Anthony Sikes and Patrick Dunne, (ADD) of Sustainability Directorate, Soldier System Command, U.S. Army Natick Research, Development and Engineering Center, Natick, MA. The work was funded under the project (DJ10) titled "Antimicrobial effectiveness of ultra-high hydrostatic pressure and pulse electric field in combination with bacteriocins for use in food-preservation," DJ10: C-DAAK60-93-K-0003.

Mssrs. Kalchayanand's and Ray's research was designed to ascertain the following: (1) do UHP or EP treatments to pathogenic and spoilage gram-positive and gram-negative bacterial cells induce sublethal injury; (2) do these sublethal injured cells develop susceptibility to antibacterial peptide of bacteriocins; (3) do UHP or EP treatments in combination with bacteriocins increase greater viability loss of pathogens and spoilage bacteria, and (4) do lysozyme in combination with UHP or EP treatments and bacteriocin enhance viability loss of these bacteria.

The research, which was divided into three phases, was initiated on 1 Oct 93. This report summarizes results from Phase II, which ended 30 Sept 95.

DESTRUCTION OF SPOILAGE AND PATHOGENIC BACTERIA BY HYDROSTATIC PRESSURE AND ELECTROPORATION IN COMBINATION WITH BIOPRESERVATES

PHASE II

Introduction

Thermal processing methods are commonly used in food industry to destroy both spoilage and pathogenic bacteria in order to increase shelf-life and assure food safety. Heat treatment, however, adversely changes flavor, taste and nutrients of food. Several new techniques are being investigated as potential alternatives to the conventional heat treatment due to the increasing consumer demand for minimally processed foods. Hydrostatic pressure (HP) and pulsed electric field (PEF) are two such novel food processing technologies. By using hydrostatic pressure or electroporation (EP; a form of PEF), protein denaturation and destruction of natural flavors and heat sensitive nutrients associated with the conventional thermal process are avoided (19, 3). Several HP processed foods have been marketed in Japan (fruit-based products), France (orange juice) and the USA, avocado spread (5).

Successful preliminary results on application of PEF to fluid foods such as orange juice and milk were also reported (9). Both HP and EP destabilize the structural and functional integrity of microbial cytoplasmic membrane (3, 16, 12) causing cell death and sublethal injury (18).

Several bacteriocins of lactic acid bacteria have been shown to be bactericidal to gram-positive bacteria, as well as to sublethally injured gram-positive and gram-negative bacteria (17, 23, 24, 25). Increased antimicrobial efficiency of HP and EP treatments in combination with bacteriocin-based biopreservative (BP) has been reported (18). Limited studies also revealed that HP and EP increased antimicrobial efficiency in combination with low heat treatment, low pH, lysozyme, chitosan or carbon dioxide (6, 7, 20).

The objectives of this study were to determine: (a) the effectiveness of both HP and EP treatments on the sublethal injury of pathogenic and spoilage bacteria and (b) the increased bactericidal efficiency of HP and EP in combination with bacteriocin-based biopreservative, lysozyme and/or low heat.

METHODS AND MATERIALS

Bacterial strains and cell preparation

Three pathogens, Listeria monocytogenes Scott A, Escherichia coli O157:H7 strain 932, and Salmonella typhimurium ATCC 14028, and two spoilage bacteria, Leuconostoc mesenteroides Ly and Lactobacillus curvatus Lb23 from our collection, Dept. of Animal Science, U. of Wyoming, were used. L. monocytogenes, E. coli and S. typhimurium were grown in Tryptic Soy Broth (Difco, MI) supplemented with 0.6% yeast extract (TSBYE) for 16 to 18 h at 37°C. L. mesenteroides and L. curvatus were grown in lactobacilli MRS broth (Difco, MI) for 16 to 18 h at 30°C. The cells were harvested by centrifugation (Beckman, CA) at 7,000 x g for 10 min at 4°C, washed and resuspended to obtain 10^6 to 10^7 cells per ml in 0.1% peptone water. The cell suspensions were maintained at 4°C before and after HP and EP treatments and prior to enumeration of colony-forming units (CFU).

Enumeration of viable and injured cells

To determine the level of the viable and injured cells in a population, a cell suspension was serially diluted and surface plated simultaneously on prepoured plates of tryptic soy agar (Difco, Detroit, MI) supplemented with 0.6% yeast extract (TSBYE) and a selective agar specific for species (Modified Oxford agar medium, MOX) of L. monocytogenes, Violet Red Bile [VRBA; Difco, MI] for E. coli, and Xylose-Lysine Deoxycholate [XLDA; Difco, Detroit, MI] for S. typhimurium. The plates were incubated at

37°C for 48 h, and CFU per ml were enumerated. L. mesenteroides and L. curvatus were enumerated on MRS agar (nonselective medium) and MRS supplemented with 5% sodium chloride (selective medium). Plates were incubated at 30°C for 48 h prior to enumeration of CFU.

Biopreservative preparation

Bacteriocin-based biopreservative (BP) was prepared, standardized for activity units (AU) as previously described (1) and chilled at 4°C before using.

Lysozyme preparation

Lysozyme hydrochloride (SPA, Bio SPA Division; purified grade) was dissolved in deionized water at the concentration of 0.04g/ml. The solution was membrane filtered through 0.45 μm low protein binding syringe filter (Gelman Sciences, Ann Arbor, MI) and chilled at 4°C before using.

HP treatment

Duplicate small plastic vials (Cryovial; Simport Plastic, Quebec, Canada; 2 ml capacity) in duplicate were filled completely with a bacterial suspension. When necessary, bacteriocin based biopreservative was added to a final concentration of 3,000 activity unit (AU) per ml. Lysozyme was added at the concentration of 100 μg/ml. The vials were individually vacuum sealed in plastic bags. Then the vials were

put into the liquid in the pressure chamber (4 by 10 in.; 10.16 by 25.4 cm) of the hydrostatic pressure unit (Engineer Pressure System, MA). Hydrostatic pressure fluid (95% water and 5% oil) was pumped into the chamber until the desired pressure was reached, held for the desired time and then released to drop the pressure to atmospheric pressure (14.7 lb/in^2). The vials were removed and stored at 4°C, and CFU per ml were enumerated within 2 h.

Pulse electric field (PEF) treatment

An ElectroSquarePorator System T820 (BTX, CA) was used for the EP treatment. Cell suspensions (200 μl) were placed in cuvettes ,0.1 or 0.2 cm. When required, bacteriocin based biopreservative and/or lysozyme were added to cell suspensions as described previously to a final concentration of 3,000 AU and 100 μg per ml, respectively. Cuvettes were kept in an ice-bath for 5 min before electroporation. Electroporation was done at either at 10μs for 3 pulses or 10μs for 10 pulses. The samples were stored in an ice prior to enumeration of CFU.

RESULTS AND DISCUSSION

Effect of electric pulse (EP) on viability loss of pathogenic and spoilage bacteria

The CFU in cell suspensions of S. typhimurium, E. coli, L. monocytogenes, L. cuvatus and L. mesenteroides before and after EP treatment were enumerated on either TSBYE or MRS agars to determine the levels of viability loss at different level of field strength (Table 1). Viability loss of all species increased with the increase in field strength. Viability loss, estimated from $\log_{10}$ colony forming unit before and after the treatments, ranged from 0.1 to 1 $\log_{10}$ when cells were electroporated at 15 kV/cm for 10μs and 3 pulses (Table 1). However, a sharp increase in viability loss of 1 to 4.6 $\log s_{10}$ was observed when cell were electroporated at 30 kV/cm for 10μs and 10 pulses. Pothakamury et al. (22) reported that 4 to 5 $\log s_{10}$ of viability loss were achieved when bacterial cells were electroporated at 16 kV/cm and 60 pulses with a pulse duration range from 200 to 300 msec. This suggests that the lethal effect of EP depends on the energy level and treatment time, pulse duration and number of pulses. Hülsheger and Niemann (13) and Hülsheger et al. (14, 15) also described the lethal effect of PEF to be a function of field strength and applied time. At 30 kV/cm, $\log_{10}$ viability loss differed with bacterial species. S. typhimurium lost viability by 4.6 $\log s_{10}$ while L. monocytogenes lost viability by 1.0 $\log_{10}$ (Table 1). L. monocytogenes, which have very small cell size as compared to

Table 1. Bactericidal effectiveness of electroporation on spoilage and pathogenic bacteria.

Bacterial strain	$\log_{10}$ CFU/ml at field strength[a] (kV)				
	0	9.0	12.0	15.0	30.0
L. mesenteroides Ly	8.8	8.6	8.1	8.0	6.1
L. curvatus FM1	8.1	8.1	8.0	8.0	4.8
E. coli O157:H7, 932	7.7	7.6	7.5	7.2	5.4
S. typhimurium ATCC 14028	10.0	9.7	9.2	9.0	5.4
L. monocytogenes Scott A	7.5	7.2	6.9	6.8	6.5

[a] All species were electroporated at the pulse duration of 10μs for three pulses from 9 to 15 kV/cm while at 30 kV/cm the pulse duration was 10μs for 10 pulses.

other bacterial species (26), was the least susceptible to EP. It appears that the susceptibility of bacteria to EP not only depends on species but perhaps also on cell size (28).

Effect of EP in combination with biopreservative and lysozyme

The field strength of 15 kV/cm and 3 pulses with a pulse duration of 10 μs caused 1 $\log_{10}$ reduction of S. typhimurium (Table 1). Therefore, we used these conditions to determine the combined effect of EP, biopreservative and lysozyme on the viability loss. The cell suspensions were subjected to EP in the presence of BP and lysozyme. EP alone caused viability loss of S. typhimurium, E. coli, L. mesenteroides and L. monocytogenes by 1, 0.5, 0.8 and 0.7 $\log s_{10}$, respectively (Figure 1). EP in combination with BP and lysozyme, however, increased viability loss to 3 $\log_{10}$ for Salmonella, 2.2 $\log_{10}$ for Escherichia, 1.6 $\log_{10}$ for Leuconostoc and 1.9 $\log s_{10}$ for Listeria (Figure 1). EP destroys bacterial cells due to the electrical field-induced rupture of the cell wall (16). EP also involves in pore formation in the biological membrane (4). The damages to wall and membrane may enchance passage of BP through the pores and cause more viability loss of bacterial cells.

Effect of HP on viability loss and injury of pathogenic bacteria

The cell suspensions of E. coli, S. typhimurium and L. monocytogenes were subjected to HP from 20,000 to 70,000 lb/in^2 for 5 min at 25°C and viable cells were enumerated on both TSBYE

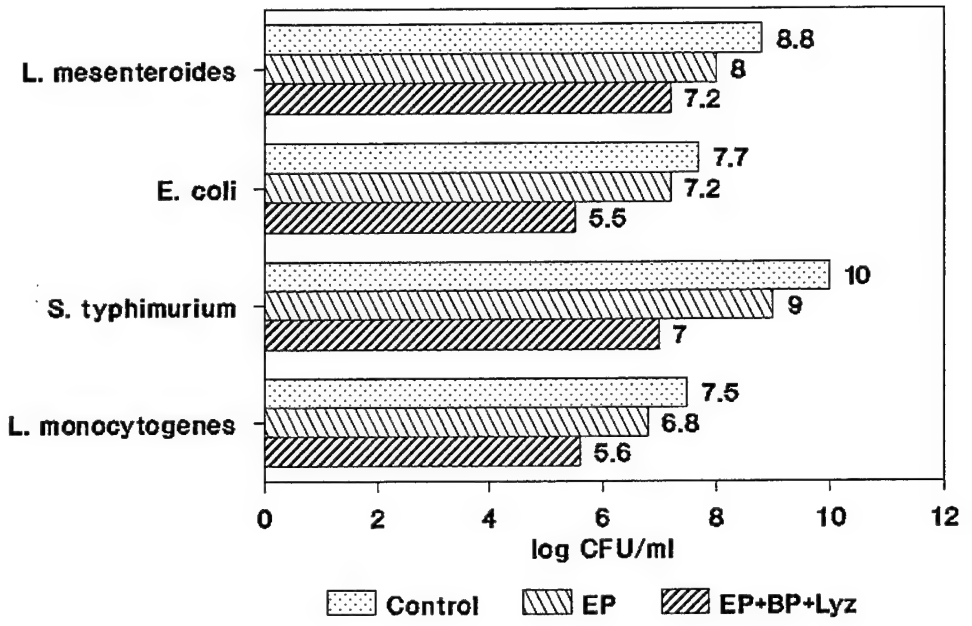

Figure 1. Bactericidal effectiveness of a combination of electroporation, biopreservative and lysozyme on spoilage and pathogenic bacteria.

and selective media to determine the levels of viability loss and sublethal injury among the survivors. The viability loss and sublethal injury of three pathogens increased by increasing pressure (Fig. 2, 3 and 4). Almost no reduction in viability and injury occurred when pathogens were pressurized at 20,000 lb/in^2. A lower degree of inactivation on bacterial cells at low pressure was reported by others (21, 27). At 30,000 lb/in^2, all pathogens had at least 1 log_{10} viability loss. It seemed that pressure-induced viability loss and injury varied from species to species. For example, at 50,000 lb/in^2, the viability loss of E. coli, S. typhimurium and L. monocytogenes was 8.9, 5.6 and 7.4 $logs_{10}$ while the injury was 0.7, 3.4 and 0.7 $logs_{10}$, respectively.

At 30,000 lb/in^2, both viability loss and injury increased gradually when pressurization time increased from 0 to 30 min (Fig. 5, 6 and 7). The degree of pressure sensitivity increased with longer exposure time. Pressurization for 30 min decreased the viability by 1.7, 3.1 and 3.3 $logs_{10}$ for E. coli, S.typhimurium and L. monocytogenes, respectively. S. typhimurium had the most sublethal injury among the three pathogens when cells were pressurized at 30,000 lb/in^2 for 30 min (Fig. 6). A high level of injury of S. typhimurium was also observed at higher pressures (Fig. 3). It appears that both viability loss and sublethal injury of E. coli, S. typhimurium and L. monocytogenes are dependent on the extent of pressure and time.

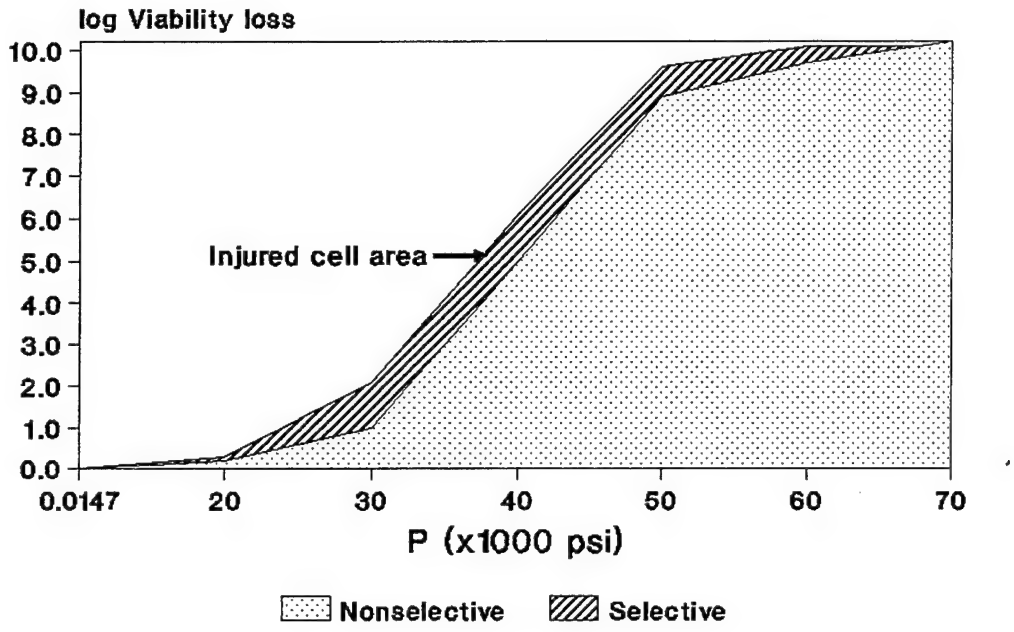

Figure 2. Viability loss and injury of Escherichia coli O157:H7 subjected to hydrostatic pressure at 25°C.

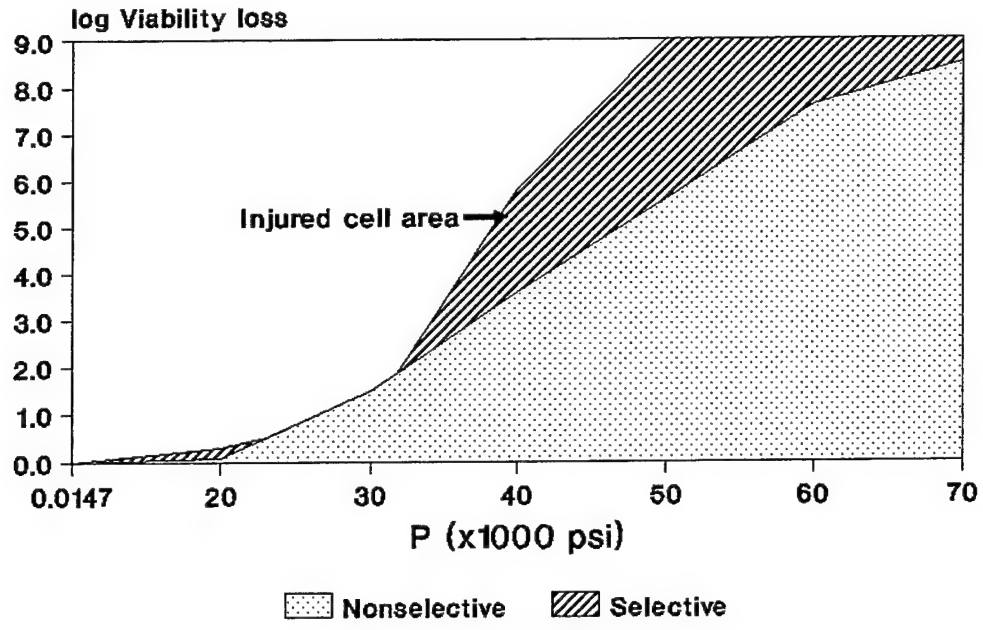

Figure 3. Viability loss and injury of Salmonella typhimurium ATCC 14028 subjected to hydrostatic pressure at 25°C.

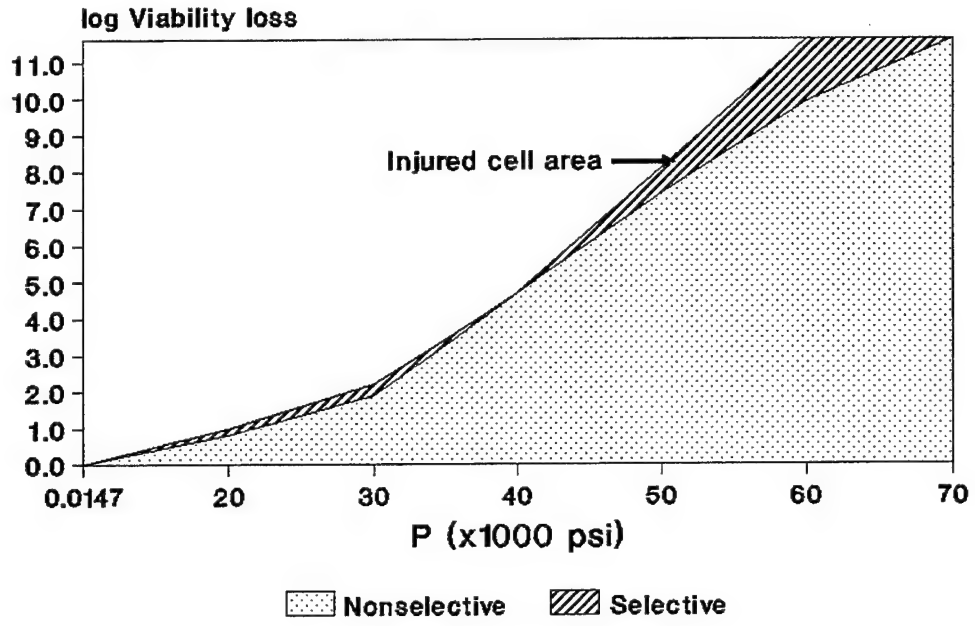

Figure 4. Viability loss and injury of Listeria monocytogenes Scott A subjected to hydrostatic pressure at 25°C.

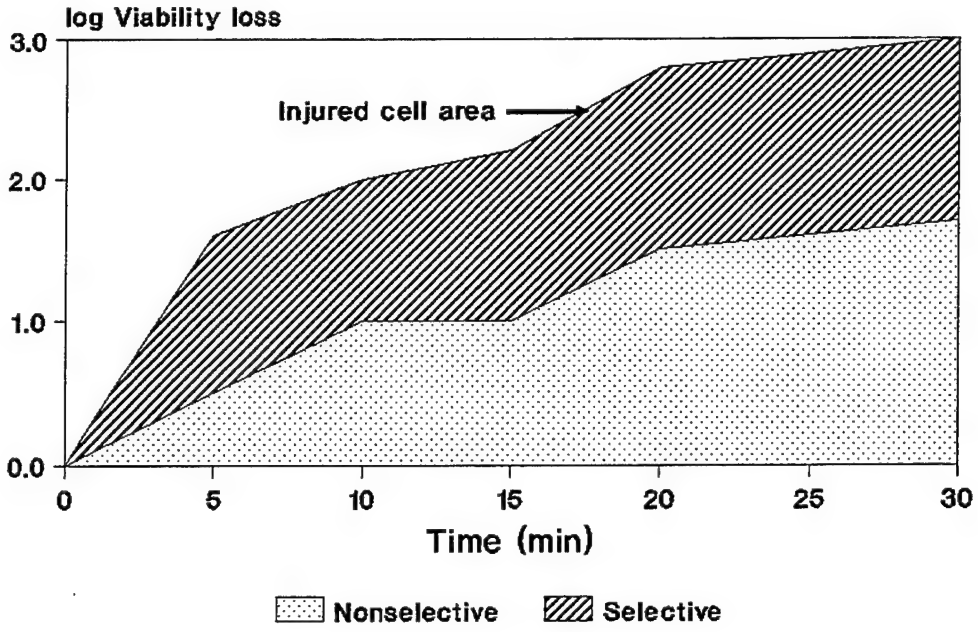

Figure 5. Viability loss and injury of Escherichia coli O157:H7 subjected to hydrostatic pressure (30,000 lb/in^2) at 25°C.

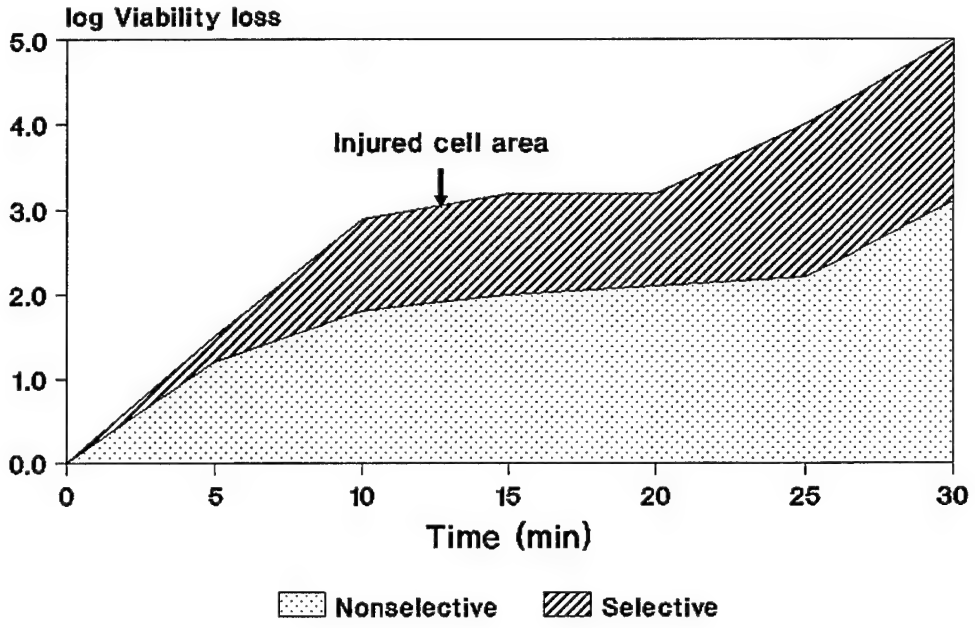

Figure 6. Viability loss and injury of Salmonella typhimurium ATCC 14028 subjected to hydrostatic pressure (30,000 lb/in^2) at 25°C.

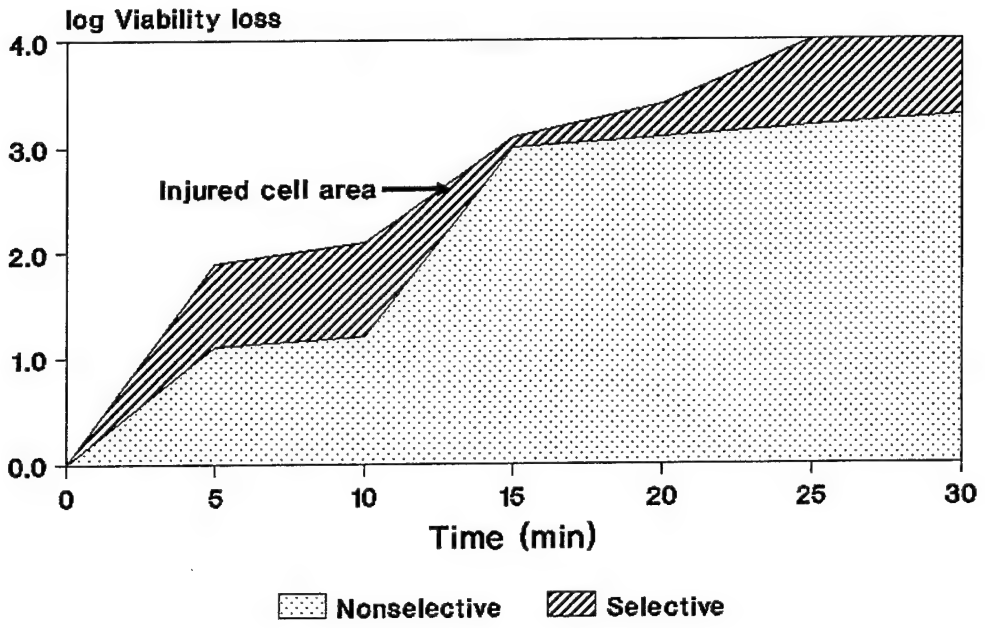

Figure 7. Viability loss and injury of Listeria monocytogenes Scott A subjected to hydrostatic pressure (30,000 lb/in^2) at 25°C.

Effect of HP and mild heat treatment on viability loss and injury of pathogenic bacteria

The combination of HP and heat treatment has been reported to be effective against the spore germination of Bacillus species (2 and 11). To determine the effect of combined pressure and mild heat treatment on viability loss and injury, E. coli and L. monocytogenes were used to represent gram-negative and gram-positive bacteria, respectively. The cell suspensions were pressurized at 30,000 lb/in^2 for 5 min at 25, 30 or 35°C. The number of cells (log_{10} CFU/ml) in the nonselective medium and the differences in number of cells between nonselective and selective medium represented viability loss and sublethal injury, respectively due to treatments. At 25 and 30°C, E. coli population was reduced by 0.6 and 0.7 log_{10} (Table 2). Viability loss increased to 1.7 log_{10} when cells were pressurized at 35°C. Similarly, sublethal injury increased when cells were pressurized at higher temperature. At 35°C, sublethal injury of E. coli increased by 2 $logs_{10}$ (Table 2). Both viability loss and injury of L. monocytogenes increased when temperature increased from 25 to 35°C (Table 3). The viability loss reached 2.4 log_{10} and sublethal injury reached 1 log_{10} when cells were pressurized at 30,000 lb/in^2 at 35°C as compared to control. It seems that mild heat treatment in combination with HP induced a greater sublethal injury to the sensitive bacterial cells.

Table 2. Effect of hydrostatic pressure and temperature on viability loss and injury of *E. coli* O157:H7

Treatment[a]	Number of cells (log_{10} CFU/ml)		
	In medium[b]		Dead and injured after treatment[c]
	Nonselec	Selective	
Control	9.9	9.8	0.0 and 0.1
HP_{25}	9.3	8.2	0.6 and 1.1
HP_{30}	9.2	8.2	0.7 and 1.0
HP_{35}	8.2	6.2	1.7 and 2.0

[a] Control was the initial cells before subjecting to HP.
HP_{25} was cells subjected to 30,000 lb/in^2 at 25°C.
HP_{30} was cells subjected to 30,000 lb/in^2 at 30°C.
HP_{35} was cells subjected to 30,000 lb/in^2 at 35°C.

[b] The nonselective medium was TSBYE and the selective medium was violet red bile agar (VRBA).

[c] The differences in numbers of CFU in TSBYE before and after treatment were considered to indicate the number of dead cells and the differences between TSBYE and VRBA after treatment were considered to indicate the number of injured cells among the survivors.

Table 3. Effect of hydrostatic pressure and temperature on viability loss and injury of L. monocytogenes Scott A

Treatment[a]	Number of cells (log CFU/ml)		
	In medium[b]		Dead and injured after treatment[c]
	Nonselective	Selective	
Control	12.2	12.1	0.0 and 0.1
HP_{25}	10.4	9.0	1.8 and 1.4
HP_{30}	10.1	8.8	2.1 and 1.3
HP_{35}	9.8	8.8	2.4 and 1.0

[a] Control was the initial cells before subjecting to HP.
HP_{25} was cells subjected to 30,000 lb/in^2 at 25°C.
HP_{30} was cells subjected to 30,000 lb/in^2 at 30°C.
HP_{35} was cells subjected to 30,000 lb/in^2 at 35°C.

[b] The nonselective medium was TSBYE and the selective medium was modified oxford agar (MOXA).

[c] The differences in numbers of CFU in TSBYE before and after treatment were considered to indicate the number of dead cells and the differences between TSBYE and MOXA after treatment were considered to indicate the number of injured cells among the survivors.

Effect of HP in combination with temperature, biopreservative, and lysozyme on viability loss and injury of pathogenic bacteria

Bacteriocins of lactic acid bacteria were bactericidal to some gram-positive bacteria and to sublethally injured gram-positive and gram-negative bacteria (17, 23,24, 25). HP induced sublethal injury to bacterial cells and viability loss of bacteria increased dramatically when HP is combined with bacteriocins (18). To determine a combined effect of HP, temperature, biopreservative and lysozyme on viability loss and injury, both E. coli and L. monocytogenes were pressurized at 30,000 lb/in^2 in the presence of bacteriocin based biopreservative and lysozyme at 25, 30 or 35°C. Viability loss and injury of E. coli increased in the presence of biopreservative and lysozyme (Table 4). HP at 25°C did not reduce E. coli population (Table 2). However, more than 4 $\log_{10}$ viability loss occured when the cells were subjected to HP at 25°C in the presence of biopreservative and lysozyme (Table 4). A similar result was observed with L. monocytogenes. Viability loss increased from 1.8 to 2.4 $\log_{10}$ as the temperature was increased from 25 to 35°C (Table 3). However, in the presence of biopreservative and lysozyme, more than 10 $\log_{10}$ reduction in viability occurred when cells were pressurized at 35°C (Table 4). Higher viability loss of L. monocytogenes than E. coli may be due to sensitivity of L. monocytogenes to bacteriocins and the hydrolysis of the ß-1,4-glycosidic bond in the peptidoglycan of gram-positive bacteria by lysozyme (8).

Table 4. A combination effect of hydrostatic pressure, temperature, biopreservative and lysozyme on viability loss and injury of pathogens

Bacterial strain	Treatment[a]	Number of cells (log CFU/ml)		
		In medium[b]		Dead and injured after treatment[c]
		Nonselective	Selective	
E. coli O157:H7, 932	Control	9.9	9.8	0.0 and 0.1
	HP_{25}+BP+Lyz	5.8	5.3	4.1* and 0.5
	HP_{30}+BP+Lyz	5.6	5.0	4.3 and 0.6
	HP_{35}+BP+Lyz	5.6	4.7	4.3 and 0.9
L. monocytogenes Scott A	Control	12.1	12.1	0.0 and 0.1
	HP_{25}+BP+Lyz	3.8	0.3	8.4 and 3.5
	HP_{30}+BP+Lyz	2.7	0.3	9.5 amd 2.4
	HP_{35}+BP+Lyz	1.3	0.0	10.9 and 1.3

[a] Control was the initial cells before subjecting to HP.
HP_{25} was cells subjected to 30,000 lb/in^2 at 25°C.
HP_{30} was cells subjected to 30,000 lb/in^2 at 30°C.
HP_{35} was cells subjected to 30,000 lb/in^2 at 35°C.
Biopreservative (BP) and lysozyme (Lyz) were added at the final concentration of 3,000 Au/ml and 100 μg/ml, respectively.

[b] The nonselective medium was TSBYE and the selective medium was either violet red bile agar (VRBA)or modified oxford agar (MOXA).

[c] The differences in numbers of CFU in TSBYE before and after treatment were considered to indicate the number of dead cells and the differences between TSBYE and selective media after treatment were considered to indicate the number of injured cells among the survivors.

CONCLUSIONS

Both HP and EP treatments caused viability loss and sublethal injury to cells of the bacterial species studied. HP treatment under mild temperature also induced a greater sublethal injury to the bacterial cells. The degree of viability loss and sublethal injury, however, varied between species and with treatments. Both intensity of treatment and time of exposure are important in determining the bactericidal effect by HP and EP. Because of the sensitivity of injured cells to biopreservative(s) and/or lysozyme, an increase in viability loss occurs when they are included in the HP or EP treatments. The inactivation of bacteria by either HP or EP is probably the result of a combination of factors. The optimum combination of HP or EP, time, temperature, biopreservative(s) and other antibacterial compounds can be used to maximize bactericidal efficiency and enhance the safety and shelf-life of foods.

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